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13. ABSTRACT ***(Maximum 200 Words)***

The goal of this research is to develop epidermal growth factor receptor (EGFR) nuclear medicine breast cancer imaging agents. Our approach is to synthesize small molecule inhibitors of the EGFR tyrosine kinase (tk) suitable for labeling with single photon or positron-emitting radioisotopes and evaluate the imaging potential of these new molecules. We have synthesized and fully characterized fourteen (14) quinazoline compounds. Most compounds inhibit EGFR tk phosphorylation activity in the nanomolar range. All compounds tested exhibited specificity for the EGFR tk versus the ErbB2 and ErbB4 tyrosine kinases. We developed a radiometric binding assay and found that the quinazolines exhibit nanomolar binding affinity for the EGFR tk ATP binding site. The octanol/water partition coefficients (Log P) lipophilicity, of the new compounds ranged from 2.8-5.5. Four compounds have been labeled with fluorine-18. Biodistribution of three of the labeled compounds in EGFR overxpressing tumor bearing mice demonstrated tumor uptake but highlighted delivery and metabolism issues. Based on our preliminary results to date, this class of labeled compounds remains promising as a source of EGFR imaging agents for breast tumors.

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Introduction

The goal of this research is to develop nuclear medicine imaging agents, to aid in the early detection, diagnosis and treatment of breast cancer, based on the epidermal growth factor receptor (EGFR) biomarker. EGF receptors are upregulated in 45% of breast tumors and tumors possessing these receptors fail to respond to conventional treatment correlating with decreased patient survivability. Our approach is to design and synthesize small molecule inhibitors of the EGFR tyrosine kinase (tk) suitable for labeling with single photon and positron-emitting radioisotopes and evaluate the imaging potential of these new molecules. The initial evaluation includes the full chemical characterization of the new molecules, an assessment of their lipophilicity, determination of their ability to inhibit EGFR tk activity and measurement of their affinity for the EGFR tk. Those compounds which possess suitable potency and binding characteristics will be radiolabeled and subjected to a secondary evaluation in in vitro cell studies and in vivo distribution studies in tumor bearing mice. The expected outcome of this research is the development of one or two radiolabeled tracers to be carried forward to preclinical EGFR imaging.

Body

Task 1: Months 1-24: Synthesize and characterize new epidermal growth factor tyrosine kinase inhibitors.

We have synthesized the eight new quinazoline analogs shown on the right hand side of Scheme 1. These compounds continue the series that we synthesized last year. They all contain a fluorine molecule and thus could be ultimately labeled with fluorine-18. They were synthesized from the common intermediates 4-chloro-6,7-dimethoxy- and 4-chloro-6,7-diethoxy-quinazoline, **1a** and **1b**, respectively.

The synthesis of the eight quinazoline analogs from the common intermediates was quite facile. The appropriate aniline or benzylamine was added to a dimethylformamide solution of **1a** or **1b** and heated for several hours. In many cases the product crystallized out of solution during the reaction. The isolated yields from this series of reactions ranged from 65-90%. All products and intermediates were purified and were chemically characterized by NMR, mass spectroscopy, melting point and elemental analysis.

Task 2: Months 3-27: Perform in vitro assays to determine tyrosine kinase affinity and inhibition. Measure the lipophilicity of the inhibitors.

Mouse BaF3 hematopoietic cells that ectopically express either EGFR, ErbB-2 or ErbB-4 were pretreated with the kinase inhibitors. Subsequently, the cells were stimulated with EGF (for cells expressing EGFR) or Neuregulin (for cells expressing ErbB-2 and ErbB-4). The cells were lysed and analyzed for receptor tyrosine phosphorylation by immunoprecipitation with anti-EGFR or anti-ErbB antibodies followed by immunoblotting with an anti-phosphotyrosine antibody. Inhibition of receptor phosphorylation at the 50 % level is reported in Table 1. Additionally, the inhibition of cellular DNA synthesis by the EGFR inhibitors was performed in human mammary tumor MCF-10A cells. The cells were incubated for 48 hours with varying concentrations of the EGFR inhibitors. Inhibition of DNA synthesis was identified by the lack of incorporation of tritiated thymidine into the DNA. The concentration

of each inhibitor that inhibits 50% incorporation of the thymidine into the DNA is reported in Table 1. These assays were performed by Dr. David Riese at Purdue University.

Almost all of the quinazoline analogs are potent inhibitors of EGFR tk phosphorylation. Most of the compounds exhibit nM inhibition. The exceptions to this are compounds **5a**, **5b**, and **10b**. The specificity of the compounds for the EGFR tk was demonstrated by the fact that the inhibition of ErbB2 and ErbB4 tk phosphorylation was 10-70 fold less than EGFR tk. In an attempt to find a less labor-intensive assay that would be predictive of EGFR tk inhibition, we measured the inhibition of DNA synthesis in EGF responsive MCF 10A cells. In our initial subset of the analogs we found that there was a good correlation between the inhibition of DNA synthesis and EGFR tk phosphorylation. However, as more compounds were tested we found that the DNA synthesis inhibition did not significantly correlate with the EGFR tk phosphorylation. Thus, the DNA synthesis assay is not a good predictor of the potency of these compounds.

We developed an EGFR tyrosine kinase receptor binding assay. Receptor binding of the quinazoline compounds was determined by a competitive radiometric assay using [^{125}I]-3'-iodoanailino-6,7-dimethoxy quinazoline as the radioligand. Commercially available A431 human carcinoma cell membrane homogenate was the EGFR source (Receptor Biology, Beltsville, MD). By varying the concentration of the quinazoline analog we were able to determine the inhibition of specific binding at the 50% level. The IC_{50} values for 10 of the quinazoline compounds are given in Table 1.

All of the compounds tested bind to the EGFR tk with high affinity. The fluorobenzyl and fluoro-trifluoromethylanilino dimethoxy compounds, **9a** and **10a**, exhibit the lowest affinities. In most cases the affinity of the diethoxy compound is higher than the corresponding dimethoxy compound. This is analogous to the reported relationship between the diethoxy and dimethoxy compounds in terms of their inhibition of EGFR phosphorylation activity (diethoxy > dimethoxy) (Bridges et al., 1997). For this initial set of data we find a significant correlation ($p<0.01$) between the receptor binding and the EGFR tk phosphorylation inhibition, as we hypothesized. The binding assay is also more useful as a predictor for good imaging agents and is much less cumbersome to perform.

The lipophilicity of the quinazoline analogs was determined by measuring the octanol/water partition coefficients (Log P) for each of the compounds. The HPLC method of Minick, et al. (1993) was used to determine the Log P (Log [octanol/water]) experimental values. The Log P values are given in Table 1. The predicted Log P values determined using the Sci LogP software from Scivision (Burlington, MA) are also included in the table. The experimental values range from 2.87 for **2a** to 5.49 for **10b**. The predicted Log P values range from 2.96 for **2a** to 4.11 for **8b**. The predicted values are smaller than the experimental values in most cases. Both sets of data correlate very well and predict the relative changes in lipophilicity between any two compounds as would be expected based on the physical differences between the two molecules. In general Log P values can be used to predict the non-specific binding of the compounds to non-receptor sites. Thus, a compound with a higher Log P might be taken up and remain in non-target tissues increasing the background activity and blurring the image. The best compounds are generally in the range of 2.5-3.5, however we will not know the overall effect of the lipophilicity on the non-specific binding of these new compounds until we look at the biodistribution of the radiolabeled compound in small animal models.

Task 3: Months 6-36: Label receptor tracers with fluorine-18, carbon-11, iodine-123, iodine-125, bromine-76. Optimize the synthetic routes for efficient production of high yield, high specific activity tracers.

We synthesized four fluorine-18 labeled compounds. The syntheses are shown in Schemes 2 and 3. The route to the labeled compounds is analogous to the synthesis of the cold compounds shown in Scheme 1. Either the labeled benzylamine **13** or the labeled aniline **17** is coupled to the appropriate chloroquinazoline, **1a** or **1b**, to give the desired labeled compound. The synthesis of the fluoroaniline was based on previous work by Feliu, et al. All of the radiolabeled quinazoline compounds were purified by HPLC and corresponded in retention time to the non-radioactive analogs. All compounds were produced in good yields.

Task 4: Months 9-36: Evaluate uptake of labeled tracers in tumors possessing differing EGFR titer ranging from 0 to over expression. Determine specific and non-specific binding.

No new progress to report on this task.

Task 5: Months 18-36: Evaluate whole body distribution in normal and tumor-bearing nude mice. Non-radioactive inhibitor will be coinjected to demonstrate receptor mediated uptake. Tumors from the same cell lines as used in the in vitro will be examined. Receptor titer in the tumor samples will be measured by in vitro assay on frozen tumor samples post dissection and correlated with the tracer uptake..

We have initiated the study of the distribution of these compounds in mice bearing tumors that overexpress EGFR. Three of the four fluorine-18 labeled compounds produced under Task 3 have been studied in this model. The choice of compounds was based on the in vitro data gathered under Task 2. The distribution of the compounds is shown in Figures 2-4.

All three compounds demonstrated some uptake in the tumor over the 2 hour study. In all cases there appeared to be washout of the label from the tumor. The blood values were low for the two fluoroanilino compounds, **18a** and **18b**. The clearance from the blood was most evident for compound **14b**. The only tumor to blood ratio that increased from 1 to 2 hours was that of the fluorobenzyl compound **14b** which indicates retention of the compound in the tumor. Whether or not this represents receptor mediated retention remains to be determined. Tumor to muscle ratios for **18a** and **18b** were less than 1 at all time points whereas the tumor to muscle ratios of **14b** were greater than 2 to 1 at both time points. High bone uptake was seen for **18a** and **18b** indicating metabolic defluorination. This is the first time that this type of metabolism has been seen with a fluorine-18 labeled compound, however, this type of metabolic defluorination of para-fluoro anilines has been described in the literature (Rietjens et al., 1989; Scribner et al., 1982; Westrop et al., 1966). The percent of the injected dose recovered from these animals was very low, on the order of 2-5%. This would indicate that the compound is being cleared very rapidly from the body through the liver and kidney so that it is not available to bind go to the tumor and bind to the receptors.

Key Research Accomplishments

- We have synthesized and fully characterized 14 quinazoline analogs.
- Most of the analogs inhibited epidermal growth factor receptor tyrosine kinase activity at pico- or nano-molar concentrations.
- Analogs tested were selective for the EGFR tk over the ErbB2 tk and the ErbB4 tk.
- We developed an EGFR tk binding assay and determined the affinity of 10 of the compounds.
- Eight out of ten analogs exhibited high affinity for the EGFR tk receptor.
- We found significant correlation between binding affinity and inhibition of receptor phosphorylation.
- The lipophilicity of the new analogs ranges from 2.8-5.5.
- Successfully labeled four analogs with fluorine-18.
- Biodistribution of radiolabeled compounds highlighted delivery and metabolism issues.

Reportable Outcomes

- Abstract presented at California Breast Cancer Research Symposium, September 1999 "Development of PET imaging agents for breast cancer" – Appendix 2.
- Abstract presented at 219th American Chemical Society National Meeting, March 2000 "Development of positron labeled epidermal growth factor receptor tyrosine kinase inhibitors: PET probes for breast cancer" – Appendix 3.
- Abstract presented at DoD Era of Hope Meeting, June 2000 "Development of radiolabeled epidermal growth factor receptor inhibitors. New probes for tumor imaging" – Appendix 4.
- Revised manuscript in press in the Journal of Labeled Compounds and Radiopharmaceuticals entitled "Synthesis of 4-(3'-[125I]iodoanilino-6,7-dialkoxyquinazolines: Radiolabeled epidermal growth factor receptor tyrosine kinase inhibitors" – Appendix 5.

Conclusions

Over this past year we have begun to gather enough data about these compounds to begin to recognize some key patterns that will assist in choosing an EGFR imaging agent. While the compounds we have developed demonstrate appropriate affinity and specificity for the EGFR, the compounds do not possess the desired distribution characteristics. The compounds may be too lipophilic to cross the cell membrane and as we saw in the case of the 4-fluoroanilino compounds, **18a** and **18b**, metabolic defluorination led to high bone uptake.

In the third year of this project we will continue to gather the key information on these compounds that we have already synthesized. This data will be invaluable in the further development of an EGFR imaging agent. In Figure 5 we show some other compounds that we

will test as potential imaging agents. Compounds **19a** and **19b** will be produced to see if moving the fluorine from the para to the ortho position eliminates the metabolic defluorination. Also the predicted Log P values for these compounds are 2.52 and 3.07 for **19a** and **19b**, respectively. These are lower than any compound synthesized to date and may also facilitate more material to cross into the tumor cells. Compound **20** will be developed based on a recent report by the Parke-Davis group (Smaill et al., 2000) pending the results of the metabolic defluorination. The 6-acrylamides have been shown to irreversibly bind to the ATP binding site of the EGFR tk. Additionally, the acrylamide would further lower the lipophilicity of the molecule to a predicted value of 2.41 that may enhance its cellular uptake.

Our biodistribution data corroborates the results from the clinical trials of the first quinazoline EGFR inhibitors, mainly that the compound clears rapidly from the body minimizing its potential therapeutic efficacy. The information that we are gathering about the distribution properties of these molecules may be very important for the future development of tyrosine kinase inhibitors as cancer therapeutics not only for the EGFR but also for ErbB2 and VEGF receptors. Therefore, our studies of the irreversible inhibitors may demonstrate whether these too might be good therapeutic candidates.

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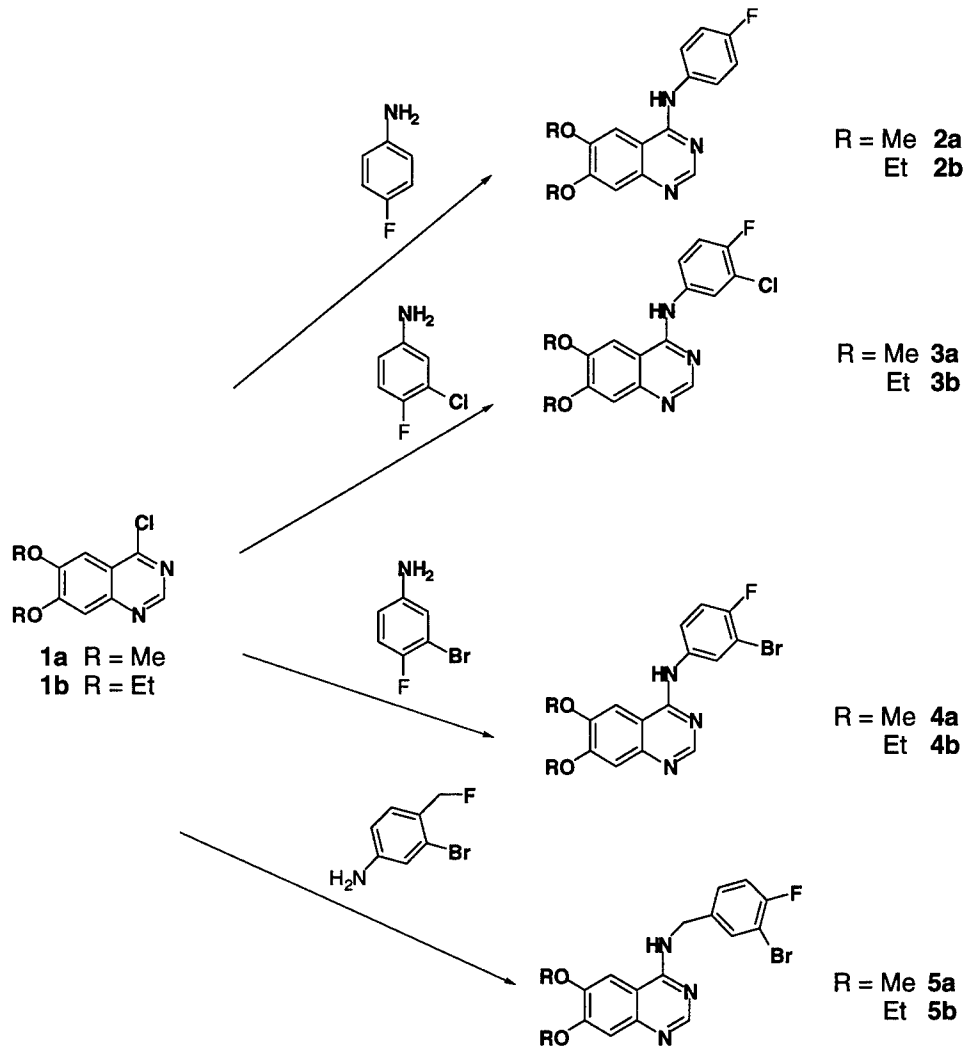
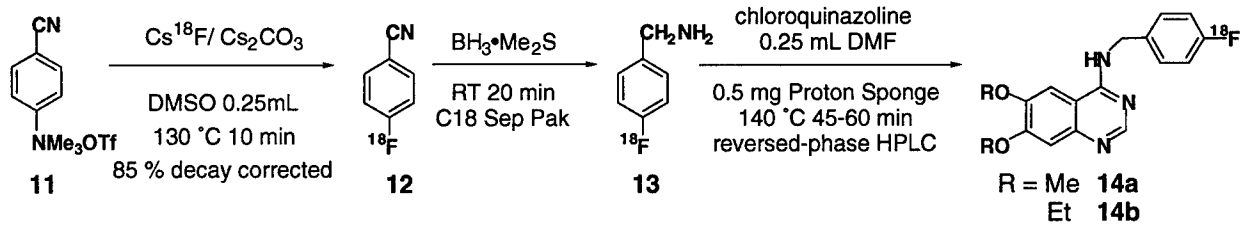
Appendix 1.

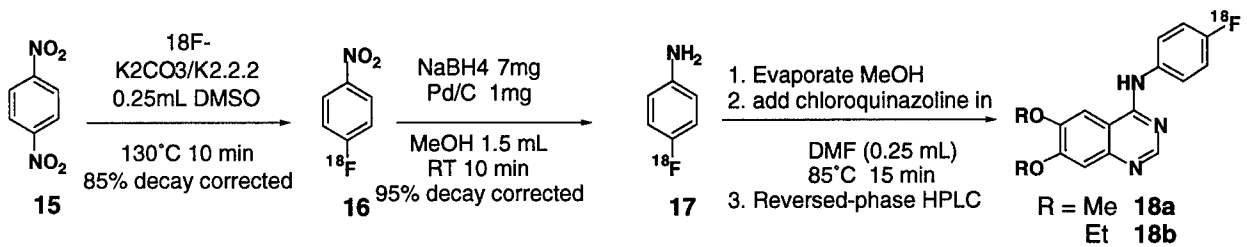


Scheme 1. Synthesis of new 4-analinoquinazolines and 4-benzylaminoquinazolines

Appendix 1. cont.



Scheme 2. Synthesis of 4-(4'-[^{18}F]fluorobenzylamino)-6,7-dimethoxy- and 4-(4'-[^{18}F]fluorobenzylamino)-6,7-diethoxy- quinazoline



Scheme 3. Synthesis of 4-(4'-[^{18}F]fluoroanalino)-6,7-dimethoxy- and 4-(4'-[^{18}F]fluoroanalino)-6,7-diethoxy- quinazoline.

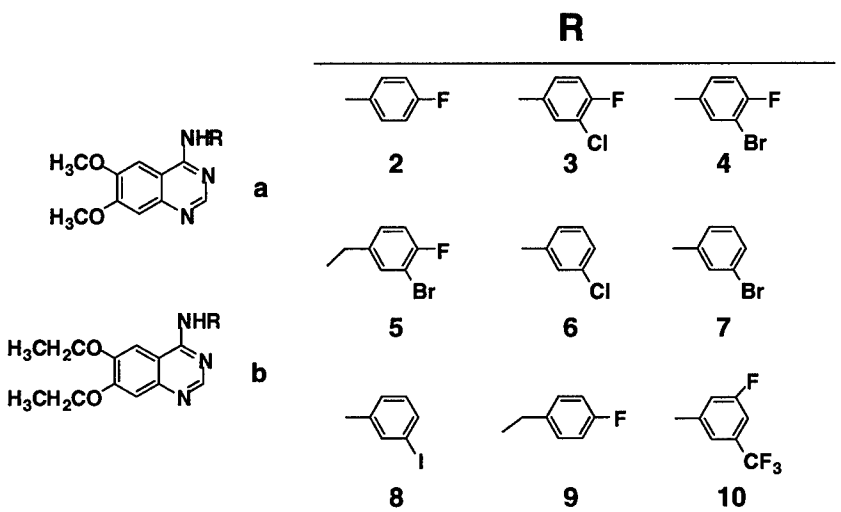


Figure 1. Quinazoline analogs synthesized in this project

Appendix 1. cont.

Table 1. In vitro data for the EGFR tk inhibitors

	Receptor Binding (IC_{50} nM)[a]	Receptor Phosphorylation (IC_{50} nM)[b]			DNA Synthesis (IC_{50} nM)[c]	Lipophilicity	
Compound	**EGFR tk**	**EGFR tk**	**ErbB2 tk**	**ErbB4 tk**	**MCF-10A**	**Log P (exptl)[d]**	**Log P (pred)[e]**
2a		12.8 ± 3.5			179 ± 31	2.87	2.96
2b		14.3 ± 2.1			141 ± 17	3.73	3.53
3a		6.1 ± 0.5			135 ± 32		3.54
3b		4.9 ± 0.4			128 ± 23		4.01
4a		6.2 ± 2.3			118 ± 16	3.86	3.29
4b		5.1 ± 1.1			98 ± 16	4.73	3.73
5a		112.2 ± 19.9			402 ± 85	4.05	3.32
5b		47.0 ± 8.7			425 ± 103	4.72	3.86
6a	1.0	0.8 ± 0.2			173 ± 24	3.51	3.64
6b	0.41	1.2 ± 0.2			108 ± 8	4.31	4.01
7a	0.25	2.1 ± 0.3	143 ± 52	49 ± 16	318 ± 61	3.49	3.38
7b	0.39	3.2 ± 0.8	215 ± 87	50 ± 19	78 ± 12	4.40	3.69
8a	1.5	11.1 ± 3.7			585 ± 108	3.65	3.76
8b	0.64	4.6 ± 2.0	69 ± 10	59 ± 29	153 ± 25	4.62	4.11
9a	34.0	10.9 ± 2.8			489 ± 87	3.02	3.05
9b	11.0	6.6 ± 1.6	231 ± 92	>100	188 ± 25	3.78	3.73
10a	35.0	19.1 ± 2.9			1634 ± 100	4.66	3.53
10b	10.0	>50			>3000	5.49	4.06

a. IC_{50} values for EGFR tk binding. Values are an average of 2-5 experiments per compound. The standard deviation is ± 10-25%.
b. IC_{50} values for tyrosine kinase phosphorylation. Values are an average of 3-10 experiments per compound.
c. IC_{50} values for DNA synthesis. Values are an average of 3-10 experiments per compound.
d. Lipohilicity of the quinazoline analogs as determined by measurement of the octanol/water (P) partition coefficient using an HPLC method. ±5-10%
e. Lipophilicity predicted by the Scivision software Sci Log P.

Appendix 1. cont.

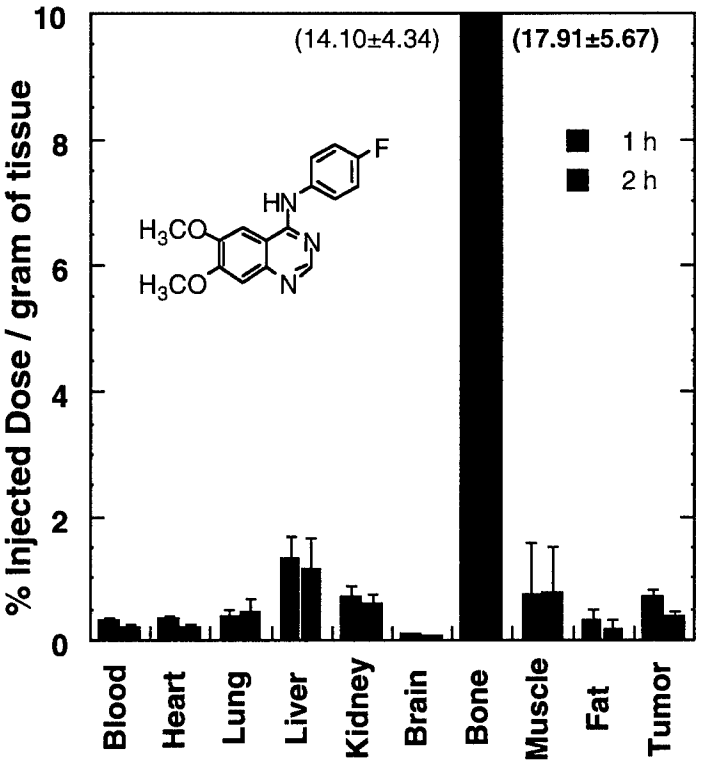


Figure 2. In vivo distribution of **18a** in MDA-468 tumor bearing mice.

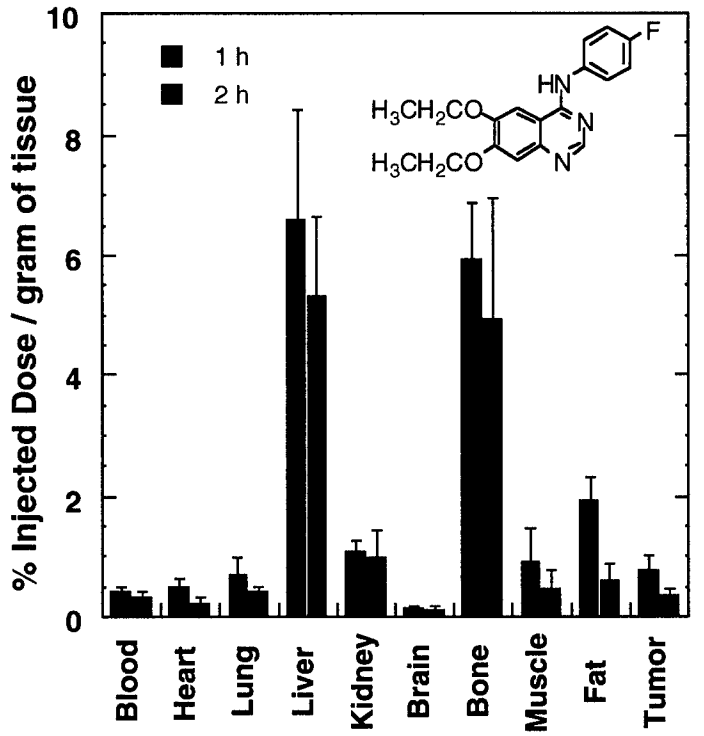


Figure 3. In vivo distribution of **18b** in MDA-468 tumor bearing mice.

Appendix 1. cont.

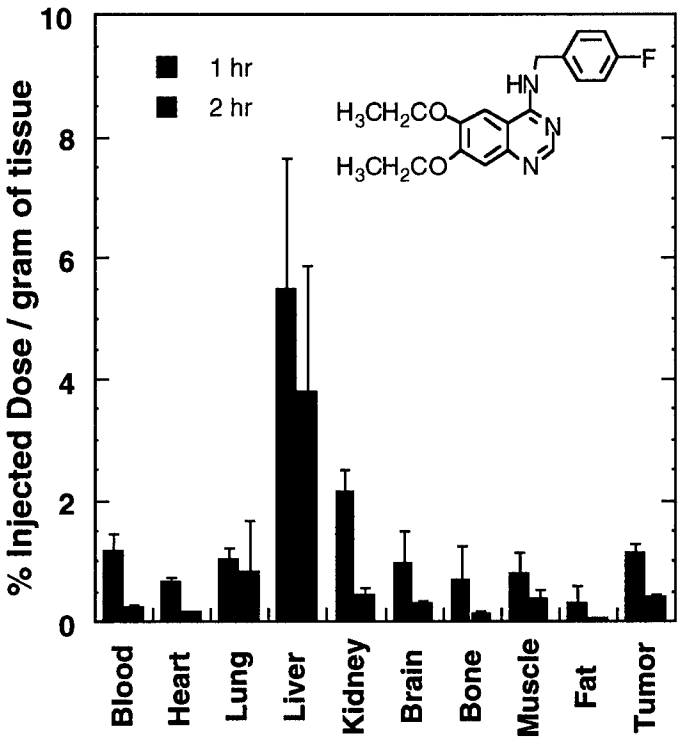


Figure 4. In vivo distribution of **14b** in MDA-468 tumor bearing mice.

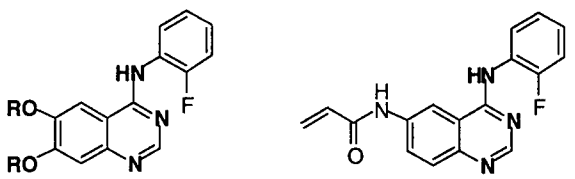


Figure 5. New EGFR imaging agents.

Appendix 2.

Abstract Submitted to Program Officials

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You have submitted the following abstract to the Spring 2000 ACS National Meeting. Receipt of this notice does not guarantee that the submission was necessarily complete or correct; imply that any errors have been detected; or indicate that it has been accepted for presentation. Additional materials, such as a preprint, may be required by the division.

Development of positron labeled epidermal growth factor receptor tyrosine kinase inhibitors: PET probes for breast cancer

Henry F. VanBrocklin[1], Andrew R. Gibbs[1], Stephen M. Hanrahan[1], Darren L. Hom[1], John K. Lim[1], and David J. Riese, II[2]. (1) Center for functional imaging, Lawrence Berkeley National Laboratory, 1 Cyclotron Road, Berkeley, CA 94720, phone: 510-486-4083, hfvanbrocklin@imasun.lbl.gov, (2) Department of medicinal chemistry and molecular pharmacology, Purdue University, West Lafayette, IN 47907

Epidermal growth factor receptor (EGFr) expression is upregulated in 45% of breast cancers. Over expression of these receptors has been correlated with poor patient prognosis as well as poor response to hormonal therapy. Inhibitors of the EGF receptor tyrosine kinase (tk) are target drug candidates for antitumor therapy. Several classes of compounds, including 4-analinoquinazolines, have been shown to inhibit EGFr tk phosphorylation, the first step in the signal transduction pathway. We have synthesized several analogs of 4-(3´-bromoanilino)-6,7-dimethoxyquinazoline (PD153035), a compound which has demonstrated potent inhibition of EGFr tk activity, suitable for labeling with fluorine-18 or carbon-11. Screening assays have demonstrated the potent inhibition of EGFr tk phosphorylation as well as the cytotoxicity of these new ligands. We will describe the synthesis, the inhibition and cytotoxicty results along with the preliminary efforts towards the synthesis of the radiolabeled probes.

Appendix 3.

Abstract for the California Breast Cancer Research Symposium

September 17-18, 1999 Los Angeles, CA.

Development of PET Imaging Agents for Breast Cancer

Henry F. VanBrocklin, John K. Lim. Center for Functional Imaging, Lawrence Berkeley National Laboratory, Berkeley CA.

Abstract:

The aim of this project is to develop new radioactively labeled pharmaceuticals (radiopharmaceutical) for the early detection and diagnosis (staging) of breast cancer using nuclear medicine imaging methods such as positron emission tomography (PET). The nuclear medicine procedure involves the injection of a radiopharmaceutical that is attracted to and preferentially accumulates in the breast tumor. The patient is then placed in an imaging device called a scanner to show the physician where the radiopharmaceutical has gone. In this case we are looking for accumulation in tumors that have a special receptor called the epidermal growth factor receptor. Only 45% of breast cancers have epidermal growth factor receptors, however, tumors with these receptors are harder to treat and do not respond to conventional hormonal treatment. Therefore, it is important that the earlier these tumors are detected the sooner any treatment can begin. Nuclear Medicine imaging agents can provide information about the tumor faster than biopsy, is less invasive than a biopsy and is about as harmful as an X-ray.

We have produced several new chemical compounds that have demonstrated an ability to bind to the epidermal growth factor receptor. We will report our latest efforts on the development of the chemistry that will be used to introduce the radioactive label into the these compounds.

Nuclear medicine techniques have a positive impact on the reduction of human and economic costs of breast cancer. These techniques are non-invasive and can provide localization information as well as some clinical evaluation of the type of disease in a single procedure, thus reducing the need for separate detection and biopsy procedures. This has both human and economic benefits by providing reliable, fast detection and diagnostic information. This in turn can have a positive impact on survivability by fostering rapid treatment response.

Appendix 4.

DEVELOPMENT OF RADIOLABELED EPIDERMAL GROWTH FACTOR RECEPTOR INHIBITORS. NEW PROBES FOR TUMOR IMAGING.

Henry VanBrocklin[1], David Riese II[2], John Lim[1], Andrew Gibbs[1], Stephen Hanrahan[1], Darren Hom[1], Michele Ono[1], and Scott Taylor[1]

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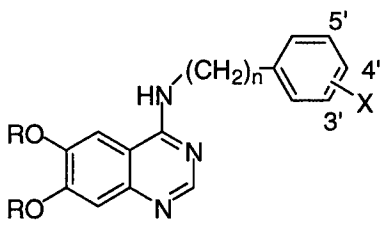
The oncogenic transmembrane epidermal growth factor receptors (EGFr) are upregulated in a variety of human neoplasms including lung, endometrial and breast tumors. The present investigation explores the development of small molecule EGFr-based imaging agents for breast cancer, where receptor upregulation correlates with poor therapeutic response and prognosis. We have synthesized ten quinazoline analogs suitable for radiolabeling, based on the known structure activity relationships of compounds developed at Parke-Davis as therapeutic agents. We have evaluated their ability to inhibit EGFr tyrosine kinase phosphorylation, using EGFr ectopically expressed in BaF3 hematopoetic cells, as well as their ability to inhibit DNA synthesis in an EGF dependent cell line, MCF-10A cells (see table below). All of the compounds tested inhibit the tyrosine kinase phosphorylation. We have identified a significant correlation between the inhibition of EGFr phosphorylation and inhibition of DNA synthesis ($p<0.005$). The DNA synthesis assay is less cumbersome, thus more attractive for high throughput screening of new inhibitors and may be more indicative of therapeutic potency. We have labeled compounds **3** and **8** with iodine-125 and demonstrated receptor-mediated uptake using MB468 cell membranes. Compounds **5** and **10** have been labeled with positron emitting fluorine-18. Initial evaluation in tumor bearing mice showed slight retention in the tumor 2 hours post injection. Based on these initial results, labeled compounds from this series of EGFr inhibitors show potential as imaging agents to detect EGF receptors in breast tumors.



analog #	n	R	X	EGFr Phos. (nM)	DNA Inhibition (nM)
1	0	Me	3'-Cl	Not Detd	Not Detd
2	0	Me	3'-Br	2.2 ± 0.4	226 ± 32
3	0	Me	3'-I	11.1 ± 3.7	585 ± 108
4	0	Me	3'-F,5'-CF_3	19.1 ± 2.9	1634 ± 100
5	1	Me	4'-F	10.9 ± 6.8	489 ± 87
6	0	Et	3'-Cl	Not Detd	Not Detd
7	0	Et	3'-Br	3.3 ± 0.9	74 ± 18
8	0	Et	3'-I	4.6 ± 2.0	159 ± 28
9	0	Et	3'-F,5'-CF_3	>50	>3000
10	1	Et	4'-F	6.6 ± 1.6	211 ± 29

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Appendix 5.

SYNTHESIS OF 4-(3'-[^{125}I]IODOANILINO)-6,7-DIALKOXYQUINAZOLINES: RADIOLABELED EPIDERMAL GROWTH FACTOR RECEPTOR TYROSINE KINASE INHIBITORS

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SUMMARY

The preparation of two radioiodinated analogs of the epidermal growth factor receptor tyrosine kinase (EGFrTK) inhibitor PD153035 (4-(3'-bromoanilino)-6,7-dimethoxyquinazoline) are reported herein. The two analogs, 4-(3'-[^{125}I]iodoanilino)-6,7-dimethoxyquinazoline and 4-(3'-[^{125}I]iodoanilino)-6,7-diethoxyquinazoline, were synthesized via iododestannylation of the corresponding 4-(3'-trimethylstannylanilino)-6,7-dialkoxyquinazolines to form the desired I-125 labeled products in good yield, high radiochemical purity (>99%) and high specific activity.

KEY WORDS: epidermal growth factor receptor, iodine-125, PD153035, quinazoline, tyrosine kinase inhibitor.

INTRODUCTION

Protein tyrosine kinases (PTKs) regulate cell division, growth and differentiation. Activation of the PTKs is one of the first steps in the signal cascade that initiates these cellular processes. The epidermal growth factor receptor (EGFr) is a member of a family of PTK-linked receptors where the tyrosine kinase domain is an integral part of the receptor. The EGFr is a 170 kD transmembrane protein possessing an extracellular ligand binding domain and an intracellular tyrosine kinase domain. The binding of EGF to the ligand domain of two adjacent receptors promotes a conformational change that brings the two receptors together. The dimerization activates the tyrosine kinase towards phosphorylation of tyrosine residues on the

adjacent receptor (interphosphorylation) as well as phosphorylation of other enzymes, thus propagating the signal throughout the cell (1-3).

The aberrant expression and activation of growth factor receptors in normal cells has been implicated in the promotion and proliferation of malignant growths (4). EGFr overexpression has been noted in a number of human neoplastic lesions (5) including lung cancer, endometrial carcinoma and breast cancer (6). Therapeutic response and patient survival has been negatively correlated with EGFr upregulation (7, 8). Small molecules capable of selective inhibition of the EGFr activation have been the target of intense research over the last several years in an effort to develop a therapeutic antitumor drug (9-11). Based on the concentration of receptors in the tumors, growth factor receptors have been sited as potential targets for imaging as well as for radiotherapeutic agents (12).

Several classes of compounds are being investigated as tyrosine kinase inhibitors (11). One class, 4-anilinoquinazolines, has been shown to be particularly potent and selective ATP site inhibitors (13). The most potent member of this class, 4-(3'-bromoanilino)-6,7-dimethoxyquinazoline (PD153035, 1), inhibits EGFr phosphorylation with a K_i of 5 pM and has demonstrated selectivity with only mM inhibition of other growth factor receptors (14).

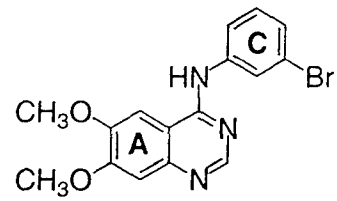

Figure 1. 4-(3'-bromoanilino)-6,7-dimethoxyquinazoline, PD153035, 1.

Several investigators have initiated programs to develop imaging agents based on the small molecule EGFr inhibitors. A number of radiolabeled analogs of PD153035 have been reported in prefatory communications. The compounds incorporate labeled substituents on the A or C rings of the anilino- or benzylamino- quinazoline (Figure 1). The C ring substituted analogs include 4-(3'-[^{125}I]iodoanilino)- (15), 4-(3'-[^{18}F]fluoro-5'-trifluoromethylanilino)- (16), 4-(3',4'-dichloro-6'-[^{18}F]fluoro-anilino)- (16), 4-(4'-[^{18}F]fluorobenzylamino)-dimethoxyquinazolines (17) and 4-(4'-[^{18}F]fluorobenzylamino)-diethoxyquinazoline (18). The 7-[^{18}F]fluoroethoxy- (19) and the 6- or 7-[^{11}C]methoxy- (19, 20) constitute the A ring labeled analogs. Preliminary in vitro studies with the 3'-[^{125}I]iodo analog demonstrated receptor mediated uptake in cells containing high EGFr titer (15). A more recent study of the ^{11}C-methoxy derivative demonstrated some uptake in human neuroblastoma xenographs in mice (21, 22). While neither of these studies were unequivocal, the evidence suggests that further studies towards the development of in vivo imaging agents for EGFr expression in tumors is warranted.

As part of an ongoing effort to develop positron-emitting EGFr imaging agents in our laboratory, we sought to produce a labeled compound for use in radiometric binding studies. To this end we report herein the detailed synthesis of two iodine-125 labeled analogs of PD153035.

RESULTS AND DISCUSSION

The two iodinated analogs presented were chosen for labeling based on known structure activity relationships derived from the inhibition of EGFr tyrosine kinase activity by numerous analogs of PD153035 produced by Parke-Davis (23). The investigators found that replacing the bromine on PD153035 with iodine increased the IC_{50} value 35 times, albeit the iodo compound still retained subnanomolar inhibition of the EGFr tyrosine kinase (IC_{50} = 0.89 nM). Replacement of the 6 and 7 methoxy moieties with 6 and 7 ethoxy groups decreased the IC_{50} of PD153035 nearly 5 fold, 0.025 nM to 0.006 nM. Based on these data we prepared 3'-iodoanalino-6,7-diethoxyquinazoline and labeled it with ^{125}I in addition to the corresponding 6,7-dimethoxy analog.

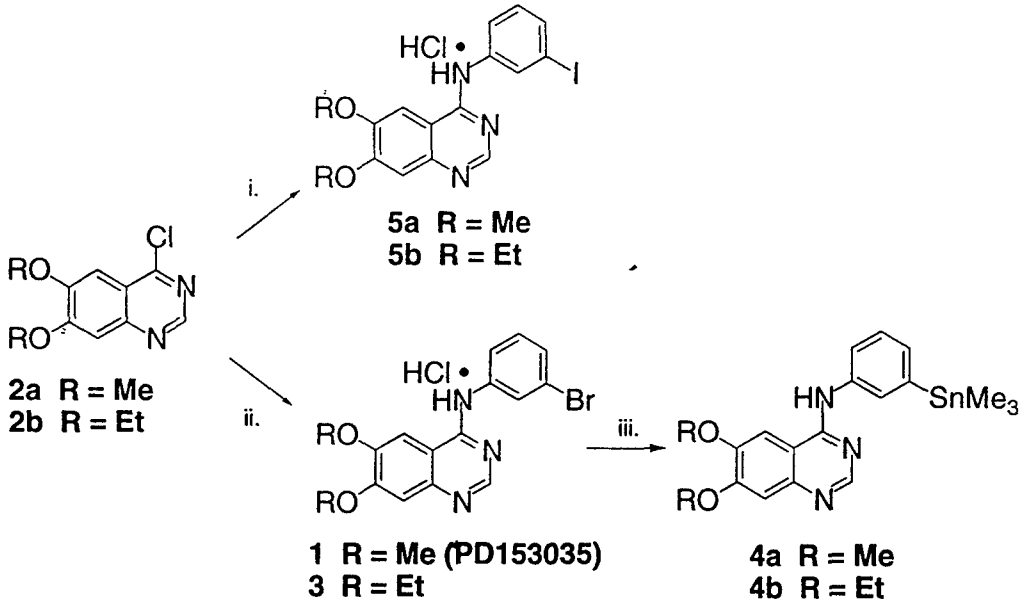

Figure 2. Synthetic routes for the iodoanilino- and trimethylstannylanilino-dialkoxyquinazolines. (i.) 3-iodoaniline, DMF, Δ; (ii.) 3-bromoaniline, DMF, Δ; (iii.) $(SnMe_3)_2$, $Pd(Ph_3)_4$, dioxane.

The synthetic route for the preparation of the analinoquinazolines is outlined in Figure 2. The common intermediates, 4-chloro-6,7-dimethoxy- **2a** and 4-chloro-6,7-diethoxyquinazoline **2b**, were used to prepare the nonradioactive iodine compounds and the trialkylstannyl precursors for labeling. The procedure outlined by Bridges *et al.* (23) was followed for the synthesis of **2a** starting from the

commercially available dimethoxyanthranilic acid. The corresponding diethoxyanthranilic acid was not commercially available; however, it was produced in one step by the saponification of the available 2-amino-4,5-diethoxy-methyl benzoate (24). The synthesis of **2b** proceeded analogously to that of **2a**.

The bromo- and iodo-anilino analogs, **1**, **3**, **5a** and **5b**, were produced by heating the appropriate bromo- or iodo-aniline with the respective chloroquinazoline, **2a** or **2b**, in anhydrous DMF. This reaction proceeded very cleanly and gave high yields (84-91%) of the haloanilino compounds. This compares favorably to the reactions reported by Bridges *et al.* (23) where they used isopropanol as the solvent. The bromoanilino compounds, **1** and **3**, were converted into the corresponding trimethylstannyl derivatives by reacting with hexamethylditin in the presence of a catalytic amount of tetrakis(triphenylphosphine)palladium(0) in 55-60% yield.

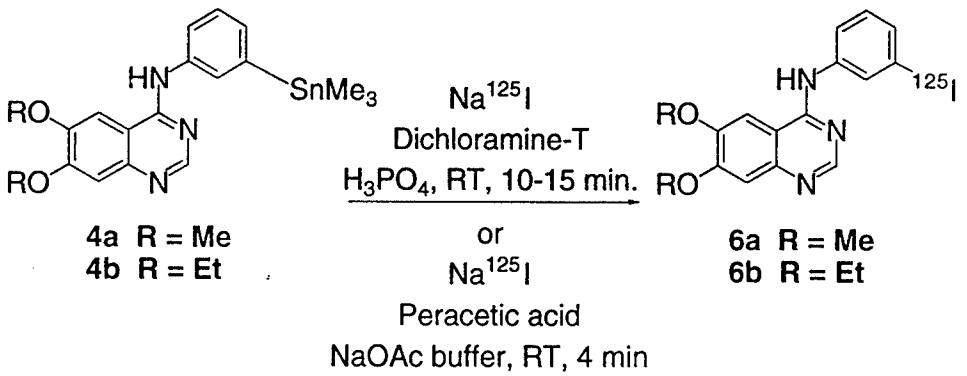

Figure 3. Radiosynthesis of 3'-[^{125}I]iodoanilino-quinazoline analogs of **6a** and **6b**.

The conversion of the stannylated compounds to the desired ^{125}I labeled quinazolines by two different methods is shown in Figure 3. The iododestannylation reaction proceeded rapidly, less than 15 minutes using dichloramine-T and less than 5 minutes using peracetic acid. The original radioidinations were carried out following an old Berkeley dichloramine-T procedure. Recently, we converted to the peracetic acid method. Purification by solid phase extraction (reversed-phase, C18) and HPLC gave either **6a** or **6b** in 40-65% radiochemical yield. The yields for the peracetic acid reactions were about 50% higher than the dichloramine-T reactions. The radiochemical purity of **6a** and **6b** was greater than 98% confirmed by normal phase TLC and coinjection with cold standard on reversed phase analytical HPLC (multiple solvent systems). Using either method, only one major radioactive product was identified on the HPLC; no other mono- or di-substituted species were formed in the reaction.

The specific activities of **6a** and **6b** ranged from 466-1900 Ci/mmol and the values were found to be independent of the method used to produce the labeled products. An HPLC chromatogram (Figure 4) of the crude **6a** dichloramine-T reaction mixture shows the separation between the radioiodinated material and the non-

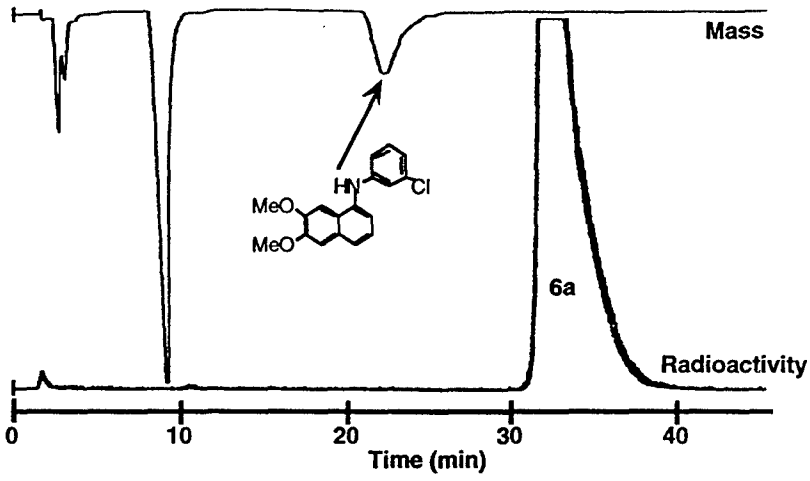

Figure 4. HPLC chromatogram of 6a produced by the dichloramine-T method. (conditions: analytical C18, 50:50 MeOH:H_2O, pH 7.4, flow 2 mL/min)

radioactive 3'-chloroanilinoquniazoline mass peak. The chloroanilinoquinazoline byproduct is not present in the peracetic acid produced radiotracer as expected. Comparing the two iodination methods, the peracetic acid reaction is more favorable for this chemistry.

EXPERIMENTAL

All chemicals were purchased from Aldrich Chemical Co. and were used without further purification. The 2-amino-4,5-diethoxy-methyl benzoate was purchased from Aldrich Specialty Chemicals. Chloroquinazolines 2a and 2b were prepared according to literature procedures (23,24). NMR spectra were obtained on a Bruker VBAMX400 400 MHz spectrometer. Elemental analyses were performed by the Microanalytical Laboratory at the College of Chemistry, University of California, Berkeley. Melting points were taken on a Mel-Temp™ apparatus and are reported uncorrected. Mass spectra were obtained on a Perkin Elmer SCIEX spectrometer at the SynPep Corporation, Dublin, CA, USA. Purification of the radioiodinated compounds was carried out by HPLC (column and conditions noted below in the synthesis section) with an in-line Linear™ UV-106 spectrophotometer to detect mass and a NaI detector and Ortec NIM components to measure the radioactivity.

4-(3'-bromoanilino)-6,7-dimethoxyquinazoline (1).

The preparation of 1 followed the method described below for 3. Yield 87%. ^1H NMR spectra were identical to those reported in the literature (23).

4-(3'-bromoanilino)-6,7-diethoxyquinazoline (3).

A clear, pale yellow anhydrous DMF solution (3 mL) of 4-chloro-6,7-diethoxyquinazoline 2b (0.1 g, 0.396 mmol) was combined with 3-bromoaniline

(64.6 μL, 0.593 mmol) to form a clear, pale pink solution. Within 15 minutes of heating the reaction flask at 80° C under argon, precipitation of a white solid was observed. The heterogeneous solution was heated for an additional 45 minutes, then cooled to room temperature for 15 minutes. The solid was filtered and washed with ethyl acetate (20 mL) to give the hydrochloride salt of **3** as a bright white, pulpy solid. Yield 0.15 g (89%). m.p. 260° C. ^{1}H NMR ($CDCl_3$): δ 8.63 (*s*, 1H, Ar*H*), 7.88 (*s*, 1H, Ar*H*), 7.87 (*s*, 1H, Ar*H*), 7.59, (*d*, 1H, Ar*H*, J = 8.0 Hz), 7.38 (*d*, 1H, Ar*H*, J = 8.0 Hz), 7.31 (*t*, 1H, ArH, J = 8.0 Hz), 4.18 (*m*, 4H, OCH_2CH_3), 1.42 (*m*, 3H, OCH_2CH_3). APCI mass spec. 388.1, 390.1 [M+1]. Elemental analysis $C_{18}H_{19}BrClN_3O_2$ calcd. C 50.90, H 4.51, N 9.89; found C 49.32, H 4.66, N 9.44.

4-(3'-trimethylstannylanilino)-6,7-dimethoxyquinazoline (4a).

The preparation of **4a** starting from 40 mg (0.11 mmol) of the free base of **1** was carried out in a manner analogous to **4b**. Yield 30 mg (61%). ^{1}H NMR ($CDCl_3$): δ 8.64 (*s*, 1H, Ar*H*), 7.73 (*d*, 1H, Ar*H*, J = 8.0 Hz), 7.62 (*s*, 1H, Ar*H*), 7.40 (*t*, 1H, Ar*H*, J = 8.0 Hz), 7.28 (*d*, 1H, Ar*H*, J = 8.0 Hz), 7.26 (*s*, 1H, Ar*H*), 7.02 (*s*, 1H, Ar*H*), 4.02 (*s*, 6H, OCH_3), 0.30 (*s*, 9H, $Sn(CH_3)_3$). APCI mass spec. 446.1, 444.0, 442.1 [M+1]. Elemental analysis $C_{19}H_{23}N_3O_2Sn$ calcd. C 51.38, H 5.22, N 9.46; found C 51.59, H 5.47 N 9.12.

4-(3'-trimethylstannylanilino)-6,7-diethoxyquinazoline (4b).

The hydrochloride salt of **3** was first converted to the free base by partitioning the solid between ethyl acetate (3 mL) and 1*N* sodium hydroxide (2 mL). After thoroughly shaking the two layers, the ethyl acetate layer was separated and the aqueous base solution was extracted with additional ethyl acetate (2 x 3 mL). The ethyl acetate fractions were pooled, dried over magnesium sulfate, filtered, and solvent removed in vacuo to give an oil, then were recrystallized from diethyl ether to give white crystals (yield 60 mg, 66%). A toluene solution (5 mL) containing the free base **3** (75 mg, 0.493 mmol), hexamethylditin (48 μL, 0.592 mmol) and a catalytic amount of tetrakis(triphenylphosphine)palladium(0) (22.3 mg, 10 mol%) was heated at 105° C under argon for 16 hours, resulting in an intense, dark black solution. The palladium catalyst was removed by eluting through a short silica pad to give a clear yellow solution. The solution was concentrated in vacuo, then purified by radial chromatography (2 mm silica plate thickness; eluted first with hexanes, then gradually adjusted to 30% ethyl acetate/hexane, and finally to 100% ethyl acetate). Purity was confirmed by a single spot in TLC (silica, 75% ethyl acetate/hexane, R_f 0.55). Yield 50 mg (56%). ^{1}H NMR ($CDCl_3$): δ 8.62 (*s*, 1H, Ar*H*), 7.75 (*d*, 1H, Ar*H*, J = 8.0 Hz), 7.61 (*s*, 1H, Ar*H*), 7.39 (*t*, 1H, Ar*H*, J = 8.0 Hz), 7.27 (*d*, 1H, Ar*H*, J = 8.0 Hz), 7.23 (*s*, 1H, Ar*H*), 7.02 (*s*, 1H, Ar*H*), 4.22 (*m*, 4H, OCH_2CH_3), 1.55 (*m*, 6H, OCH_2CH_3), 0.30 (*s*, 9H, $Sn(CH_3)_3$). APCI mass spec. 474, 472.1, 470 [M+1]. Elemental Analysis $C_{21}H_{27}N_3O_2Sn$ calcd. C 53.42, H 5.76, N 8.90; found C 53.76, H 6.06, N 8.54.

4-(3'-iodoanilino)-6,7-dimethoxyquinazoline (5a).

The preparation of **5a** followed the method described above for **3**. The chloroquinazoline **2a** (0.28 g, 1.22 mmol) was reacted with 1.5 equiv. of iodoaniline to yield 0.5 g (92%) of **5a**. m.p. 252-253° C. ^{1}H NMR (DMSO): δ 7.91 (*s*, 1H, Ar*H*), 7.31 (*s*, 1H, Ar*H*), 7.16 (*s*, 1H, Ar*H*), 6.80 (*d*, 1H, Ar*H*, J = 8.0 Hz), 6.70 (*d*, 1H, Ar*H*, J = 8.0 Hz), 6.36 (*s*, 1H, Ar*H*), 6.32 (*t*, 1H, Ar*H*, J = 8.0 Hz), 3.05 (*s*, 3H, OC*H*$_3$), 3.03 (*s*, 3H, OC*H*$_3$). APCI mass spec. 408.0 [M+1]. Elemental Analysis $C_{16}H_{15}ClIN_3O_2$ calcd. C 43.31, H 3.41, N 9.47; found C 43.66, H 3.45, N 9.51.

4-(3'-iodoanilino)-6,7-diethoxyquinazoline (5b).

The preparation of **5b** followed the method described above for **5a**. The chloroquinazoline **2b** (0.12 g, 0.46 mmol) was reacted with 1.5 equiv. of iodoaniline to yield 0.19 g (88%) of **5b**. m.p. 265-266° C. ^{1}H NMR (DMSO): δ 8.86 (*s*, 1H, Ar*H*), 8.20 (*s*, 1H, Ar*H*), 8.10 (*s*, 1H, Ar*H*), 7.75 (*d*, 1H, Ar*H*, J = 8.0 Hz), 7.67 (*d*, 1H, Ar*H*, J = 8.0 Hz), 7.30 (*s*, 1H, Ar*H*), 4.27 (*m*, 4H, OC*H*$_2$CH$_3$), 1.44 (*m*, 6H, OCH$_2$C*H*$_3$). APCI mass spec. 436.0 [M+1]. Elemental analysis $C_{18}H_{19}ClIN_3O_2$ calcd. C 45.83, H 4.06, N 8.91; found C 46.11, H 3.45, N 8.85.

4-(3'-[^{125}I]iodoanilino)-6,7-dimethoxyquinazoline (6a) or 4-(3'-[^{125}I]iodoanilinio)-6,7-diethoxyquinazoline (6b).

Dichloramine-T Method: A 4 mL vial equipped with a Teflon cap and a stir bar was charged with the 3'-trimethylstannylanilino-6,7-dialkoxyquinazoline precursor **4a** (~0.5 mg, 1.1 μmol) or **4b** (~0.5 mg, 1.1 μmol) in acetonitrile (200 μL). To the vial was added a solution of Na^{125}I (~1 mCi) followed by the addition of 85% phosphoric acid (50 μL) and dichloramine-T (20 μL of a 2 mg/mL solution in acetonitrile). After reacting at room temperature for 10-15 minutes, the mixture was quenched with 10% $Na_2S_2O_5$ (50 μL). The quenched solution was diluted with 5-10 mL of deionized H_2O, then eluted through an activated C18 Sep-Pak® trapping >95% of the measured activity. The cartridge was then eluted with 0.2 mL of methanol, then an additional 1.6 mL of methanol. The second elution fraction containing the bulk of the activity was slowly evaporated under a gentle stream of argon. The residue was dissolved in 2 mL of a buffered methanol/water solution (phosphoric acid/triethylamine) and chromatographed by HPLC (Waters μBondapak™ C18 column, 3.9 x 300 mm, 50% MeOH/water final pH 7.40, flow = 2 mL/min, retention time **6a**: 25-34.5 min or **6b**: 53-70 min.). The product was concentrated by trapping on an activated C18 Sep-Pak®. **6a** (0.5 mCi, 53% yield) or **6b** (0.45 mCi, 45% yield) was collected in 1 mL of methanol eluent.

Peracetic Acid Method: A 4 mL vial equipped with a Teflon cap and a stir bar was charged with the 3'-trimethylstannylanilino-6,7-dialkoxyquinazoline precursor **4a** (~0.5 mg, 1.1 μmol) or **4b** (~0.5 mg, 1.1 μmol) in acetonitrile (200 μL). To this vial was added sodium acetate buffer (0.6M, pH 4.5, 100 μL) and Na^{125}I followed by peracetic acid (30 μL, Aldrich). The reaction was stirred for 4 min. at room

temperature and then quenched with 10% $Na_2S_2O_5$ (100 µL). The solution was made basic with saturated $NaHCO_3$ and transferred to a syringe. The reaction vial was rinsed with acetonitrile (100 µL) and added to the syringe along with 8 mL of water. The solution was passed through an activated C18 Sep-Pak® and the trapped radioiodinated product was eluted with 3 mL of MeOH and concentrated for HPLC purification (as noted above). The yield of **6a** and **6b** using this method was 60-65%.

Specific Activity Measurement: The purified labeled products, **6a** and **6b**, were concentrated and analyzed by reversed phase analytical HPLC (Phenomenex Bondclone™ 10 C18 column, 3.9 x 300 mm, 50-80% MeOH/water final pH 7.40, flow = 2 mL/min). The HPLC derived specific activity was calculated based on the activity injected and the mass/UV response measured relative to a standard curve.

CONCLUSION

We have successfully labeled two EGFr tyrosine kinase inhibitors with iodine-125 using two different synthetic methods. The peracetic acid method produced slightly higher yields of cleaner product in a one half to one third the time and is the preferred method for producing these compounds in the future. The compounds were produced in sufficiently high yield, high radiochemical purity and high specific activity. Both these compounds demonstrated receptor mediated affinity for the EGFr tyrosine kinase binding site in initial binding studies. One of these compounds may find utility in the radiometric binding assay we are developing for the measurement of the receptor binding affinity of novel EGFr inhibitors.

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